Protocol for the Preparation and Titration of HIV-1 Env-pseudotyped Viruses

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I. Introduction

The performance of neutralizing antibody assays under properly standardized, optimized, and validated conditions requires accurate titration of virus infectivity. Moreover, use of molecularly cloned Env-pseudotyped viruses has advantages over uncloned virus in terms of reagent stability and affording greater reproducibility and precision in neutralization assays. This protocol describes the production of molecularly cloned Env-pseudotyped viruses in 293T/17 cells by co-transfection with an Env-expressing plasmid plus a backbone plasmid containing a defective Env gene. Co-transfection generates pseudovirus particles that are able to infect cells, but due to the absence of a complete genome, they are generally unable to produce infectious progeny virions. This single round of infection is readily detectable in genetically engineered cell lines that contain a Tat-responsive reporter gene, such as luciferase. Neutralization assays that are based on a single-round of infection should use viruses that are titrated in a similar single-round infection format.

It is important to note that Env-pseudotyped viruses are handled and treated the same as live HIV-1 cultures as replication-competent virus could be present. Sequence analysis indicates that recombination can occur that repairs the defect in the backbone plasmid(s) lacking Env, thus rendering the virus replication-competent. The recombination event occurs at a relatively low frequency and the proportion of the pseudovirus that is replication-competent is low. When recombination occurs that renders replication-competent virus (RCV), the virus generated is predicted to behave identically to wild-type HIV-1. This protocol can be used to transfect other adherent cell lines such as 293S and 293S_GnTI- cells.

II. Definitions

GM: Growth Medium

DMEM: Dulbecco's Modified Eagle Medium

Luc: Luciferase

RLU: Relative Luminescence Units

FBS: Fetal Bovine Serum

ID: Identification

DPBS: Dulbecco's Phosphate Buffered Saline

TCID: Tissue Culture Infectious Dose

CO_2: Carbon Dioxide

RCV: Replication Competent Virus

EDTA: Ethylenediaminetetraacetic acid

DEAE-Dextran: Diethylaminoethyl-Dextran

PPE: Personal Protective Equipment

III. Reagents and Materials

Recommended vendors are listed. Unless otherwise specified, products of equal or better quality than the recommended ones can be used whenever necessary.

293T/17 cells
Vendor: American Tissue Culture Collection

TZM-bl cells
Vendor: National Institute of Health (NIH) AIDS Reagent Program

Complete Growth Medium (see Protocol for Reagent Preparation for Use in the Neutralizing Antibody Assay for HIV-1 in TZM-bl Cells)

DEAE-Dextran, hydrochloride, average Mol. Wt. 500,000 (see Protocol for Reagent Preparation for Use in the Neutralizing Antibody Assay for HIV-1 in TZM-bl Cells)
Manufacturer: Sigma

Trypsin-EDTA (0.25% trypsin, 1 mM EDTA)
Sterile
Manufacturer: Thermo Fisher Scientific

Trypan Blue (0.4%)
Manufacturer: Thermo Fisher Scientific

Dulbecco's Phosphate Buffered Saline (DPBS)
Sterile
Manufacturer: Thermo Fisher Scientific

FuGENE 6 or FuGENE HD Transfection Reagent
Manufacturer: Promega Corporation

Britelite™ Plus Reporter Gene Assay System (see Protocol for Reagent Preparation for Use in the Neutralizing Antibody Assay for HIV-1 in TZM-bl Cells)
Manufacturer: Perkin Elmer Inc.

NOTE 1: The lyophylized Britelite Plus substrate is not classified as hazardous.

NOTE 2: Bright Glo Luciferase Assay System from Promega Corporation is an acceptable substitute for britelite plus. Please follow manufacturer's guidelines for preparation and use. Bright Glo is classified as hazardous. Personal Protective Equipment (PPE) is required when working with these reagents.

Microliter pipettor tips
Sterile
Manufacturer: Rainin or Sartorius

Combitips, sterile
Manufacturer: Eppendorf

Disposable pipettes, sterile, individually wrapped
Vendor: Falcon/VWR
1 ml pipettes
5 ml pipettes
10 ml pipettes
25 ml pipettes
50 ml pipettes
100 ml pipettes
Manufacturer: Corning

Flat-bottom culture plates, 96-well, low evaporation, sterile
Manufacturer: Corning

Flat-bottom black solid plates, 96-well, Costar brand
Manufacturer: PerkinElmer, Inc.

Cryogenic vials, 1.5 ml sterile screw cap, frosted label
Manufacturer: Sarstedt Brand Products

Culture flasks with vented caps, sterile
Manufacturer: Corning
T-25 flask
T-75 flask

Reagent reservoirs, 50 ml capacity
Manufacturer: Corning

IV. Instrumentation

Recommended manufacturers are listed. Unless otherwise specified, equipment of equal or better quality than the recommended ones can be used whenever necessary.

Biological Safety Cabinet
Manufacturer: Baker Comp., NuAIRE

CO_2 Incubator (37ºC, 5% CO2 standard requirements)
Manufacturer: Panasonic

Centrifuge and Microcentrifuge
(low speed capable of up to 500 x g)
50 ml tube holder
15 ml holder
Microtitration plate holder
Manufacturer: Jouan

18 place standard rotor F-45-18-11 for 1.5 ml microcentrifuge tubes
Manufacturer: Eppendorf

Luminometer
Manufacturer: PerkinElmer, Inc.

Hemacytometer
Manufacturer: INCYTO

NOTE 3: An automated cell counting device (i.e. Countess, Manufacturer: Invitrogen) may be used in lieu of a light microscope / hemacytometer for cell counting and viability calculation.

Light Microscope
Manufacturer: Olympus

Pipettor
Single channel electronic pipettor, 10-300 µl
12-channel electronic pipettor, 50-1200 µl
12-channel electronic pipettor, 10-300 µl
Single channel manual, 0.5-10 µl
Single channel manual, 2-20 µl
Single channel manual, 20-200 µl
Single channel manual, 100-1000 µl
Manufacturer: Sartorius

PipetteAid XP
Manufacturer: Drummond Scientific Co.

12-channel manual pipettor, 20-200 µl
Manufacturer: Rainin

Repeater pipette
Manufacturer: Eppendorf

Automated Cell Counter
Countess Automated Cell Counter
Countess Cell Counting Chamber Slides
Manufacturer: Invitrogen

Ultra Low Temperature Freezer (-80°C or lower)
Manufacturer: Thermo Fisher Scientific

4°C Refrigerator
Manufacturer: LABRepCo, Inc.

-20°C Freezer
Manufacturer: LABRepCo, Inc.

V. Specimens

Molecularly cloned Env-pseudotyped viruses are generated by using a two plasmid system: An Env expression plasmid (e.g., pc Deoxyribonucleic acid (DNA) 3.1D/V5-His-TOPO-Env) and a backbone vector (e.g., pSG3ΔEnv) that expresses the entire HIV-1 genome except for Env.

VI. Protocol

Pseudovirus Preparation

NOTE 4: All incubations are performed in a humidified 37°C, 5% CO_2 / 95% air environment incubator unless otherwise specified.

1. Preparation of Cells

NOTE 5: This protocol can be used to transfect other adherent cell lines such as 293T, 293S and 293S_GnTI-. These adherent cell lines are maintained in T-75 culture flasks. Cell monolayers are disrupted and removed by treatment with Trypsin-EDTA at confluency.

1.1. Trypsinize the 293T/17 cells (see Protocol for Trypsin-EDTA Treatment for Disruption of Cell Monolayers) and perform viable cell count.

1.2. At least 80% of the cells must be viable in order to use the cells for transfection.

2. Transfection of 293T/17 Cells

2.1. Seed 3-8 x 10^6 293T/17 cells in a T-75 flask containing 12 ml of GM. Incubate overnight (20-24 hours). Monolayers should be 50-80% confluent on the day of transfection.

NOTE 6: The following steps pertain to 1 x T75 flask. Additional flasks can be prepared by multiplying each volume by an appropriate factor per flask.

2.2. Based on the volume of Env plasmid DNA and backbone plasmid DNA to be dispensed, add the appropriate volume of DMEM into one sterile tube, such that the total volume of the mixture is 100 µl. Dispense 4 µg of Env plasmid DNA and 8 µg of backbone plasmid DNA into the tube containing DMEM and mix well with a pipettor.

NOTE 7: The amounts of Env plasmid and backbone plasmids shown above are a recommendation. The optimal ratio of Env and backbone plasmids used to prepare each env-pseudotyped virus strain may vary. If it is necessary to improve the virus yield, the optimal ratio for a particular virus may be determined by transfecting 293T/17 cells using different Env and backbone plasmid ratios. A different ratio may be used if it generates higher virus yield than the recommended ratio.

2.3. To a second sterile tube, add 652 µl DMEM. Pipet 48 µl of FuGENE 6 reagent directly into medium without contacting the sides of the plastic tube. Mix well with a pipettor.

2.4. Transfer the entire contents of the plasmid DNA mixture from the first tube to the second tube containing the FuGENE solution. Mix by pipetting or briefly vortexing.

2.5. Incubate for 30 minutes at room temperature to allow transfection complex formation.

2.6. Using a pipette, slowly mix the entire contents of the transfection complexes immediately prior to adding them to the T-75 flask of 293T/17 cells. Gently swirl the flask for uniform distribution of the complexes.

2.7. Incubate for 3 to 8 hours at 37°C to allow the plasmids to enter the cells.

2.8. Carefully remove the medium containing DNA FuGENE complexes and replace with 15 ml of fresh GM. Incubate for 24-72 hours.

2.9. Harvest virus-containing culture supernatants by collecting the medium from the flask using a pipette. Adjust the FBS concentration in the virus-containing culture medium to 20% (i.e. for each 1 ml of virus harvested, add 0.125 ml of FBS) and mix using a pipette. Filter the virus-containing culture medium through a 0.45-micron filter. Aliquot the virus to sterile screw-cap cryogenic vials that have been labeled to identify the isolate name and date of harvest. The harvest date becomes the specific lot number. Store the aliquots at −80°C.

2.10. Record the harvest date information and location of the vials as well as the information regarding the Env plasmid DNA, backbone plasmid DNA, and cells used in the transfection in a laboratory virus log book.

2.11. Add 6-12 ml of fresh GM to the cells in each flask. Incubate overnight and harvest the virus-containing culture supernatants once more, as indicated above, and discard the cells.

NOTE 8: If virus has a historically low infectivity titer, it may be cost effective to harvest only one time, 72 hours after transfection. If performing a 3 day harvest, it is advisable to seed cells in the lower range of concentration, i.e. 3 x 10^6 cells per T-75 flask. Cell monolayers should not be more than 50% confluent before transfection.

3. Titrating Pseudovirus in TZM-bl Cells (TCID Assay)

3.1. Place 100 µl of GM per well in all wells of a 96-well flat-bottom culture plate. Add 25 µl of virus to the first 4 wells of a dilution series (column 1, rows A-D for one virus and rows E-H for a second virus). Mix the virus (at least 5 times) in column 1 and transfer 25µl to column 2. Repeat the mixing and transferring of virus through column 11 (these are serial 5-fold dilutions). After final transfer and mixing is complete, discard 25 µl from the wells in column 11. Wells in column 12 will serve as cell controls for background luminescence (no virus added). See Attachment 1 for plate layout.

3.2. Prepare a suspension of TZM-bl cells at a concentration of 100,000 cells/ml in GM as described below:

NOTE 9: Plates may be incubated in the incubator or at room temperature while cells are prepared.

3.2.1. Trypsinize the TZM-bl cells (see Protocol for Trypsin-EDTA Treatment for Disruption of Cell Monolayers) and perform viable cell count.

3.2.2. At least 80% of the cells must be viable in order to use the cells for TCID assay.

3.2.3. Cell calculations are performed (see below).

To calculate the cell concentration, multiply the average this number of cells per quadrant, the dilution factor and 10,000 to yield the cell concentration, "C1," in cells/ml. To calculate the total cell mixture volume, "V_2", that you need, multiply the number of plates by the total volume of cell mixture needed per plate. The concentration of cells desired is 100,000 cells/ml, "C_2". Thus, using the equation $C_1V_1 = C_2V_2$, one can solve for "V_1", the volume of cells needed.

For example:

Total number of cells counted = 60
Number of quadrants counted = 4
Dilution = 1:10
Number of plates = 1
Cell mixture needed per plate = 10 ml

60 cells ÷ 4 quadrants = 15 cells/quadrant

15 cells/quadrant x dilution factor of 10 x 10,000 cells/ml = 1,500,000 cells/ml = C1

1plate x 10 ml/plate = 10 ml = V_2

Optimum final concentration of cells = 100,000 cells/ml = C_2

Therefore: $C_1V_1 = C_2V_2$

V1 = (100,000 cells/ml x 10ml) ÷ 1,500,000 cells/ml = 0.67 ml of cells

3.2.4. Addition of DEAE-Dextran to Cells

NOTE 10: The concentrations of DEAE-Dextran shown will vary by batch of DEAE-Dextran. The actual optimal concentration should be determined for each new batch of DEAE-Dextran prepared by performing a titration assay (see Protocol for Determination of Optimal Concentration of DEAE-Dextran).

To calculate the amount of DEAE-Dextran to use, first multiply the optimal concentration of DEAE-Dextran (derived from the titration experiment) by 0.2 ml (the final volume in each well) to get the amount of DEAE-Dextran per well. Multiply the amount of DEAE-Dextran per well by 100 wells/plate (96 wells rounds to 100) to derive the amount of DEAE-Dextran per plate. Divide the amount of DEAE-Dextran needed per plate by the stock concentration of the DEAE-Dextran to yield the volume of DEAE-Dextran stock needed. Multiply this number by the number of plates to yield the total volume of DEAE-Dextran stock needed.

For example:

If the optimal concentration of DEAE-Dextran in the assay is 10 µg/ml and the DEAE-Dextran stock is at 5 mg/ml

10 µg/ml x 0.2 ml (volume in well) = 2 µg of DEAE-Dextran needed in each well

2 µg x 100 wells/plate = 200 µg of DEAE-Dextran needed per plate = 0.2 mg of DEAE-Dextran

0.2 mg of DEAE-Dextran per plate ÷ 5 mg/ml stock concentration = 0.04 ml of DEAE-Dextran stock needed per plate

To calculate the amount of GM to add, subtract the total volume of cells needed and the total volume of DEAE-Dextran stock needed from the total volume of cell mixture needed.

The total volume needed for one plate is 10 ml.

Therefore 10 ml – 0.67 ml cells – 0.04 ml DEAE-Dextran = 9.29 ml of GM

3.3. The GM/cells/DEAE-Dextran suspension should be prepared as follows: Add GM and DEAE-Dextran then mix; Add cells and thoroughly mix the prepared cell suspension immediately prior to plating. Dispense 100 µl of the prepared cell suspension (10,000 cells per well) to each well in rows A through H columns 1-12, starting from column 12.

3.4. Rinse the pipettor tips in a reagent reservoir containing sterile DPBS or change the pipettor tips between each plate to minimize carry-over.

3.5. Cover plates and incubate for 48 – 72 hours if Env-pseudotyped viruses are used. If replication-competent virus is used, the plates should be incubated 46-50 hours to minimize virus replication.

3.6. After incubation, remove plates from incubator. Plates should not stay out of the incubator longer than one hour before running the luciferase reaction.

NOTE 11: Using a light microscope, check the plates for syncytia and cell toxicity. Make a note on a laboratory book.

3.7. Remove 100 µl of culture medium from each well, leaving approximately 100 µl. Dispense 100 ml of Britelite Plus reagent to each well.

3.8. Incubate at room temperature for 2 minutes to allow complete cell lysis. Mix by pipettor action (at least two strokes) and transfer 150 µl to a corresponding 96-well black plate. Read the plate immediately in a luminometer (if possible, the luminometer should be connected to a secure network file server). The electronic data should be backed up on a routine basis.

3.9. Calculate the TCID using the "TCID" macro (if possible, this macro should be stored on a secure network file server). Select the pseudovirus dilution that yields approximately 50,000 to 150,000 RLU equivalents (+/- 15,000 RLU). For pseudoviruses that do not reach 50,000 RLU, select a

dose of virus that yields at least 15,000 RLU but is not toxic to the cells via light microscopy. If the virus yield does not reach 15,000 RLU, the virus will not be used.

***NOTE 12*:** The RLU equivalents measured in the TCID assay may not match the RLUs in the virus control of the neutralization plate. This difference is acceptable provided that the virus control is greater than or equal to 10 times the background (cell control) and the virus is not toxic to the cells based on light microscopy.

NOTE 13: Assays with replication-competent viruses are incubated for 48 hours to keep the virus replication to a minimum.

NOTE 14: TCID assays should be performed for pseudovirus stocks that have been thawed one time. These vials are marked with a "1X".

VII. Attachments

Attachment 1: Template for TCID Assay, 2 Viruses per Plate

	1	2	3	4	5	6	7	8	9	10	11	12
A	Dil 1	Dil 2	Dil 3	Dil 4	Dil 5	Dil 6	Dil 7	Dil 8	Dil 9	Dil 10	Dil 11	CC
B	Dil 1	Dil 2	Dil 3	Dil 4	Dil 5	Dil 6	Dil 7	Dil 8	Dil 9	Dil 10	Dil 11	CC
C	Dil 1	Dil 2	Dil 3	Dil 4	Dil 5	Dil 6	Dil 7	Dil 8	Dil 9	Dil 10	Dil 11	CC
D	Dil 1	Dil 2	Dil 3	Dil 4	Dil 5	Dil 6	Dil 7	Dil 8	Dil 9	Dil 10	Dil 11	CC
E	Dil 1	Dil 2	Dil 3	Dil 4	Dil 5	Dil 6	Dil 7	Dil 8	Dil 9	Dil 10	Dil 11	CC
F	Dil 1	Dil 2	Dil 3	Dil 4	Dil 5	Dil 6	Dil 7	Dil 8	Dil 9	Dil 10	Dil 11	CC
G	Dil 1	Dil 2	Dil 3	Dil 4	Dil 5	Dil 6	Dil 7	Dil 8	Dil 9	Dil 10	Dil 11	CC
H	Dil 1	Dil 2	Dil 3	Dil 4	Dil 5	Dil 6	Dil 7	Dil 8	Dil 9	Dil 10	Dil 11	CC

Rows A-D are for Virus 1. Rows E-H are for Virus 2. *CC, Cell control wells (cells only).*